Antibiosis Revisited: Bacteriocins Produced by Dairy Starter Cultures[1]

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ABSTRACT

Well before the existence of starter bacteria was recognized, their activities were instrumental in preserving dairy foods. During growth in fermented products, dairy starters, including lactobacilli, lactococci, leuconostocs, streptococci, and propionibacteria, produce inhibitory metabolites. Inhibitors include broad-spectrum antagonists, organic acids, diacetyl, and hydrogen peroxide. Some starters also produce bacteriocins or bactericidal proteins active against species that usually are related closely to the producer culture. Several bacteriocins have been biochemically and genetically characterized. Evaluating properties of the *Lactobacillus acidophilus* bacteriocin, lactacin B, led to a new purification protocol. Purified lactacin B migrates in SDS-PAGE as a single 8100-Da band with inhibitory activity after Coomassie blue staining. Production of lactacin B is enhanced by cultivation of the producer with the sensitive indicator, *Lactobacillus delbrueckii* ssp. *lactis* 4797; understanding this interaction may increase knowledge of production of bacteriocins in heterogeneous cultures. Bacteriocins have been recently identified in dairy propionibacteria. Jenseniin G, a bacteriocin produced by *Propionibacterium jensenii* P126, has narrow activity; propionicin PLG-1 produced by *Propionibacterium thoenii* P127 inhibits propionibacteria, some fungi, *Campylobacter jejuni*, and additional pathogens. Better understanding of these antagonists may lead to targeted biocontrol of spoilage flora and foodborne pathogens.
(**Key words**: antibiosis, bacteriocins dairy cultures, lactic acid bacteria)

Abbreviation key: LaF = lactacin F, **MWCO** = molecular weight cutoff, **ORF** = open reading frame, **Str^r** = streptomycin-resistant.

INTRODUCTION

Babel (6) defined antibiosis as "an antagonistic association between microorganisms to the detriment of one of them." Antibiosis continues to be of particular interest in fermented dairy foods, for which the antagonistic products of the growth of starter cultures help to preserve the fermented product. Within the past few years, numerous reports (1, 2, 4, 5, 10, 14, 15, 22, 25, 26, 27, 30, 32, 34, 35, 36, 37, 43, 44, 52, 58, 59, 61, 62, 64, 67, 74, 79, 85, 86, 90, 91) of inhibitors produced by dairy cultures have been published. The present review focuses on antagonistic metabolites and bacteriocins (bactericidal proteins with activity against species that are usually closely related to the producer culture) produced by dairy lactobacilli, lactococci, leuconostocs, and propionibacteria.

ANTIBIOSIS-MEDIATING AGENTS

Lactic acid bacteria used in dairy applications include the lactococci (*Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, and *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*), the lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and, occasionally, *Lactobacillus casei*), the leuconostocs (primarily *Leuconostoc mesenteroides* ssp. *cremoris*),

Received February 10, 1992.
Accepted August 14, 1992.
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TABLE 1. The pKa and inhibitory spectra[1] of organic acids produced by dairy cultures.

Acid	pKa	Organisms inhibited
Acetic	4.75	Bacteria except lactics and *Acetobacter* species; most molds
Lactic	3.9	Bacteria (at low pH)
Propionic	4.9	Molds and bacteria

[1]Source: (42).

and *Streptococcus thermophilus*. During the fermentation of dairy products, these cultures metabolize lactose to lactic acid. Acid production lowers the pH and creates an environment that is unfavorable to pathogens and spoilage organisms. In addition, the low pH of fermented foods potentiates the antimicrobial effects of organic acids. As demonstrated by Rubin et al. (72) and reviewed by Piard and Desmazeaud (65), organic acids show greater lethality to bacteria than do the inorganic acids, which dissociate completely in aqueous solutions. In a fermented food with pH approximating pKa, an undissociated lipophilic acid (acetic, lactic, or propionic acid; Table 1) dissolves in the cell membrane and diffuses into the cell. At the higher internal pH of the cell, the acid dissociates and acidifies the cell (42). The net effect is disruption of the pH gradient across the membrane and cessation of related metabolic functions that are essential to cellular survival.

Propionic acid produced by dairy propionibacteria demonstrates broader inhibition than acetic and lactic acids. Propionic acid and its sodium and calcium salts are effective antimycotics and are added to many baked products to inhibit molds. The propionates also inhibit Gram-negative bacteria but are ineffective against Gram-positive species (42).

Another antagonistic metabolite produced by many dairy starter cultures is hydrogen peroxide (Table 2). Whittenbury (95) reported that hydrogen peroxide accumulates in cultures of lactobacilli, leuconostocs, and pediococci. Anders et al. (5) observed that lactic streptococci [now designated as lactococci (78)] produced sufficient hydrogen peroxide to be autoinhibitory. Dahiya and Speck (18) identified hydrogen peroxide as an inhibitor of *Staphylococcus aureus* in cultures of *Lb. delbrueckii* ssp. *lactis* and *Lb. delbrueckii* ssp. *bulgaricus*.

Dairy cultures have been manipulated to overproduce hydrogen peroxide. Waxman et al. (93) examined lactococci for inhibitors and identified a hydrogen peroxide producer, *L. lactis* ssp. *lactis* NCDO 916. The hydrogen peroxide producer was protoplasted, and a hydrogen peroxide super producer, 916-4, was isolated from the regenerated cells. The parental culture and the super-producing mutant culture were added separately to cottage cheese, and their effects on shelf-life were compared. The mutant culture inhibited *Pseudomonas fragi* and increased shelf-life to 37 d (an increase of approximately 17%).

Dairy cultures including lactobacilli, leuconostocs, *L. lactis* ssp. *lactis* biovar. *diacetylactis*, and propionibacteria produce the

TABLE 2. Hydrogen peroxide production by lactic acid bacteria.

H_2O_2-Producing species	Inhibited culture	Reference
Lactococci, leuconostocs, lactobacilli		(94)
Lactococci	Self	(5)
Lactobacillus delbrueckii ssp. *lactis*, *Lactobacillus delbrueckii* ssp. *bulgaricus*	*Staphylococcus aureus*	(18)
Lactobacillus plantarum	*Pseudomonas, Proteus, Bacillus cereus*, and *Bacillus megaterium*	(68)
Lactobacillus acidophilus	*Staph. aureus, Escherichia coli, Salmonella typhimurium, Clostridium perfringens*	(27)
Lb. delbrueckii ssp. *bulgaricus*	Psychrotrophs	(1)
Lactococcus lactis ssp. *lactis* biovar. *diacetylactis*	*Pseudomonas fragi*	(93)

inhibitory flavor metabolite, diacetyl. Jay (43) documented the inhibitory effects of diacetyl on 4 yeasts, 10 lactic acid bacteria, 12 Gram-positive species, and 14 Gram-negative cultures. Lactic acid bacteria were insensitive to diacetyl at 100 to 400 μg/ml. Diacetyl inhibited all other cultures when the pH was neutral or acidic; inhibitory activity decreased as pH increased above 7.0. Diacetyl was most inhibitory to Gram-negative bacteria: ≤200 μg/ml were effective. Inhibition of yeasts requires 200 to 300 μg/ml of diacetyl; at least 350 μg/ml were required to inhibit Gram-positive, nonlactic species. Because leuconostocs only produce up to .2 m*M* (14 μg/ml) diacetyl (12) and because lactobacilli may produce 1.2 m*M* (84 μg/ml) diacetyl (54), diacetyl may be a minor contributor to broad-spectrum antagonism in these organisms.

BACTERIOCINS

Of particular current interest are proteinaceous mediators of antibiosis or bacteriocins produced by dairy starter cultures. Several reviews have addressed bacteriocins of lactic acid bacteria. In a 1988 review, Klaenhammer (47) discussed bacteriocins produced by dairy and nondairy species. Schillinger's review of 1990 (75) focused on bacteriocins of lactic acid bacteria used in meat systems. The review of Piard and Desmazeaud (65, 66) examined bacteriocins and other inhibitors produced by all lactic acid bacteria and was published after the presentation by Barefoot and Nettles (9). This review addresses bacteriocins of dairy lactic acid bacteria and dairy propionibacteria and emphasizes studies published since 1988.

Classical criteria (84) define bacteriocins as bacterially produced bactericidal proteins or peptides with narrow activity centered on species that are closely related to the producer culture. For bacteriocins of lactic acid bacteria, e.g., lacticin 481 (64, 67), leucocin (35, 36), and nisin (21, 41), broader activity spectra are not uncommon. Adsorption to sensitive cells (84) is not a uniform requirement for activity by bacteriocins produced by dairy cultures. Finally, numerous exceptions exist within bacteriocinogenic dairy cultures for the fifth criterion of plasmid-encoded bacteriocin production and host cell immunity (84). Therefore, Klaenhammer's (47) definition of bacteriocins as bactericidal proteins that are active against species that are usually closely related to the producer culture has been more widely accepted.

TABLE 3. Bacteriocins produced by dairy lactococci.

Lactococcus lactis subspecies	Bacteriocin	References
lactis	Nisin	(41, 53)
	Lactostrepcins	(22, 49)
	Bacteriocins	(26)
	Lacticin 481	(64, 67)
	Unnamed	(85)
cremoris	Diplococcin	(19, 63)
	Lactococcins A, B, and N	(37, 90, 91, 92)
lactis biovar. *diacetylactis*	Lactococcin A	(33, 74, 83)
	Lactostrepcins	(49)
	Bacteriocin S50	(48)

Within the past few years, much attention has been devoted to the biochemical and genetic characterization of lactococcal bacteriocins (for a partial listing, see Table 3). Since its approval as an antibotulinal additive in pasteurized, processed cheese spread in the US in 1988 (24), many studies have addressed the molecular biology of nisin, the broad-spectrum bacteriocin that contains unusual lanthionine, dehydroalanine, and dehydrobutyrine residues and is produced by *L. lactis* ssp. *lactis* (41, 53). The mode of action of nisin has been elucidated (25, 50, 73), genes encoding the nisin prepeptide have been cloned (11, 23, 46), and the steps in posttranslational processing of nisin to an active lantibiotic have been postulated (11).

Nisin causes rapid loss of amino acids and ions from sensitive cells and cytoplasmic membrane vesicles and dramatically decreases the membrane potential of whole cells (73). These data support the conclusion that the primary target of nisin is the cytoplasmic membrane. Gao et al. (25) demonstrated that nisin dissipates both the membrane potential and the pH gradient across the cytoplasmic membrane, providing confirmative evidence that nisin exerts its lethal action by disrupting cellular proton motive force. Liu and Hansen (50) attribute part of the activity of nisin to the dehydro amino acids. The hypothesis is that these dehydro amino acids act as nucleophilic

electron acceptors toward nucleophiles in the cellular target.

Three studies report the cloning of genes encoding the nisin A precursor peptide from genomic (11) or plasmid DNA (23, 46); the cloned gene sequences are identical. Subsequent reports (28, 38, 80) indicate that the nisin A prepropeptide gene is located within a polycistronic operon on the chromosome of *L. lactis* ssp. *lactis* ATCC 11454. Gireesh et al. (28) provided conclusive evidence that nisin is determined chromosomally. Matings of a nisin-producing donor with a nisin-negative recipient resulted in conjugal transfer of a 68-kb pair fragment encoding nisin production. Analysis of the genome of the donor, recipient, and nisin-producing transconjugant by pulsed-field gel electrophoresis indicated that chromosomal DNA had been transferred (28).

Nisin is considered to affect only Gram-positive species. However, Stevens et al. (81) have demonstrated that application of nisin in combination with EDTA inhibits *Salmonella* ssp. and other Gram-negative bacteria. Further studies (82) with outer membrane lipopolysaccharide mutants of *Salmonella typhimurium* indicate that deletions in the core oligosaccharide of the lipopolysaccharide destabilize the outer membrane and result in nisin sensitivity. Thus, nisin may be combined with treatments that alter the outer membrane of Gram-negative cells for inhibition of additional species of concern in food spoilage and food safety (81, 82). Probably, the activity of other membrane-active bacteriocins produced by dairy cultures may be similarly affected by chelators.

Bacteriocin-producing strains of *L. lactis* ssp. *lactis* occur at a frequency of 9% (26), and additional broad-spectrum bacteriocins have recently been detected. Thuault et al. (85) identified four strains that produce bactericidal, protease-sensitive inhibitors of vegetative cells of *Clostridium tyrobutyricum*, a species implicated in late "blowing" of Emmental-type cheeses. The bacteriocins also inhibit strains of *Strep. thermophilus* and *Lb. helveticus* but are inactive against other Gram-positive and Gram-negative genera. Piard et al. (64) identified a 5500-Da peptide, lacticin 481, that is bactericidal to most lactococci, some lactobacilli, leuconostocs, and clostridia, including *C. tyrobutyricum*. Lacticin is produced by *L. lactis* ssp. *lactis* 481 during associative growth in milk and inhibits the sensitive species *L. lactis* ssp. *cremoris* CNRZ 117. The population of the sensitive culture decreased from 1×10^6 cfu/ml to less than 100 cfu/ml within 8 h; only 20 cfu/ml were recovered at 24 h (64). These data illustrate the effectiveness of lacticin 481 in establishing producer dominance. Lacticin 481 is produced maximally in producer cultures that are maintained at pH 5.5 and has been purified to homogeneity by ammonium sulfate precipitation, gel filtration, and reversed-phase HPLC (67). The purified bacteriocin typically is a single peptide of 1.7 kDa but also appears as a dimer of 3.4 kDa (67). Like nisin (21, 41), lacticin 481 contains lanthionine and can be classified as a lantibiotic (67).

Diplococcin (19, 63) and most bacteriocins (22, 37) produced by *L. lactis* ssp. *cremoris* are active against only closely related species. Lactostrepcin 5 (las 5) produced by strain 202 (22) inhibits other lactococci by inducing leakage of K^+ ions and ATP (96). Holo et al. (37) reported that as little as 400 molecules (or 7 pmol) of the limited spectrum bacteriocin lactococcin A inhibit sensitive lactococci; prior to that study, a minimum lethal concentration had not been reported for bacteriocins of lactic acid bacteria. Holo et al. (37) purified lactococcin and isolated the gene from the producer, *L. lactis* ssp. *cremoris*. The *lcnA* gene was cloned and sequenced; analyses suggested that lactococcin A is synthesized as a 75-amino acid preprotein containing a 21-amino acid N-terminal extension that could function as a signal sequence. The *lcnA* sequence is identical to that of a bacteriocin cloned by van Belkum et al. (90, 91) from the plasmid p9B4-6 harbored by *L. lactis* ssp. *cremoris* 9B4. An identical sequence recently was reported for the plasmidborne structural gene of a bacteriocin from *L. lactis* ssp. *lactis* biovar. *diacetylactis* WM4 (83), which points to the mobility of bacteriocin determinants among lactococcal subspecies. Molecular analysis of the cloned genes from WM4 identified four complete open reading frames (ORF) implicated in the production of lactococcin A (83). The four ORF were identified as the genes for the lactococcin A immunity protein (*lciA*), the structural proteins (*lcnA*), and two proteins (*lcnD* and *lcnC*) that were similar to membrane trans-

locator proteins that are ATP-dependent, signal sequence-independent, and active in Gram-negative bacteria (83).

Interestingly, lactococcin A is only one of three bacteriocin determinants identified on the plasmid p9B4-6. Genes for lactococcin B (92) and lactococcin M (90, 91) have been identified and cloned. Placement of the lactococcin B gene under the control of a T-7 RNA polymerase-specific promoter permitted its expression in *Escherichia coli* (92). That report (92) established a precedent for the occurrence of multiple bacteriocin systems in other dairy cultures.

Strains of *L. lactis* ssp. *lactis* biovar. *diacetylactis* (Table 3) produce limited spectrum bacteriocins, including lactococcin A (33, 74, 83), the lactostrepcins (49), and bacteriocin S50 (48). Activities of these bacteriocins typically are restricted to other lactococci.

Information about bacteriocins of dairy leuconostocs is limited. Orberg and Sandine (62) isolated a contaminant in a culture of *L. lactis* ssp. *cremoris* 290PC and identified it as a *Leuconostoc* sp. The isolate, strain PO184, inhibited strains of *L. lactis* ssp. *cremoris* but did not inhibit the nisin producer, *L. lactis* ssp. *lactis* ATCC 11454 (62). Whether inhibition was due to a bacteriocin is unknown; the responsible agent has not yet been characterized. Daba et al. (14) identified a bacteriocin produced by an isolate of *Leuconostoc mesenteroides* from Cheddar cheese. Mesentericin 5 is an SDS-insensitive, heat-stable peptide (4000 Da) with activity against *Listeria monocytogenes*, *Listeria ivanovii*, *Enterococcus faecalis*, *Brevibacterium linens*, and *Pediococcus pentosaceus*, but not against leuconostocs, lactobacilli, lactococci, and Gram-negative bacteria (14).

More complete characterization of leuconostoc bacteriocins is limited to those produced by isolates from meat (2, 32, 36). The broad spectrum bacteriocin leucocin A-UAL 187 is produced by an isolate of *Leuconostoc gelidum* from fresh meat and is antagonistic to most lactic acid bacteria and single strains of *Ent. faecalis* and *List. monocytogenes* (36). Leucocin A has been purified, sequenced, and cloned; it is a 37-amino acid, 3930-Da, plasmid-encoded peptide containing a disulfide bridge between cysteinyl residues located at positions 9 and 14 (35). An operon containing the leucocin A structural gene (*leuA*) and an additional ORF was identified by hybridization with a mixed oligonucleotide probe homologous to the leucocin N-terminal sequence. The leucocin gene sequence encodes 61 amino acids, i.e., leucocin A-UAL 187 and a 24-amino acid N-terminal extension. The plasmid pJH8.6L with a 2.9-kb fragment containing the *leuA* operon was transformed into strains of *Lactococcus*, *Leuconostoc*, and *Carnobacterium*. Although the transformants contained the plasmid, they did not express leucocin activity (35).

Numerous bacteriocins are produced by lactobacilli from dairy and nondairy sources. A partial list includes fermenticin, produced by *Lactobacillus fermentum* (20); lactocin LP27 (88, 89) and helveticin J (44, 45), produced by *L. helveticus*; lactacin F (LaF) (57, 58, 59) and lactacin B (8, 9), produced by *Lb. acidophilus*; brevicin 37 (71), produced by *Lactobacillus brevis*; caseicin 80 (70, 71), produced by *Lb. casei*; lacticins A and B, produced by *Lb. delbrueckii* ssp. *lactis* (86); gassericin A, produced by *Lactobacillus gasseri* (87); plantaricin A (17) and plantacin B (94), produced by *Lactobacillus plantarum*; and sakacin A (76, 77) and lactocin S (55, 56), produced by *Lactobacillus sake*.

Rammelsberg and Radler (71) described a heat-stable (121°C, 1 h) antagonist, brevicin 37, that is produced by *Lb. brevis* B 37 and inhibitory to related lactobacilli, pediococci, leuconostocs, and *Nocardia corallina*. They (71) designated brevicin as a bacteriocin but may have done so prematurely, because they did not report assessments of bactericidal activity. In the same study (71), they described a proteinaceous antagonist, caseicin 80, produced by *Lb. casei* B. 80 that inhibited only *Lb. casei* B 109. Caseicin 80 was susceptible to treatment at >60°C for 10 min and was retained by ultrafiltration membranes with molecular size exclusion limits of 50,000 Da (71), suggesting that caseicin 80 is one of the macromolecular bacteriocins produced by lactobacilli. In a subsequent study (70), caseicin 80 was purified and further characterized. Cultivation of the producer culture in a synthetic medium supplemented with peptone dialysate and treatment with motimycin C increased caseicin 80 production five- to sevenfold (70), and represents the first phenotypically induci-

ble bacteriocin identified in lactic acid bacteria.

Toba et al. (87) reported that all 30 strains of *Lb. gasseri* isolated from infant feces produce catalase-insensitive, protease-sensitive, heat-stable (120°C, 20 min) inhibitors of other lactobacilli. The antagonists produced by *Lb. gasseri* LA 33 and 39 had identical spectra and enzymatic sensitivity and were termed gassericin A.

The bacteriocin lactocin 27 (88, 89) was identified and purified in the 1970s. Since that time, only the bacteriocin helveticin J (44) has been described in *Lb. helveticus*. Helveticin J is produced maximally in MRS broth cultures of *Lb. helveticus* NCDO 481 maintained at pH 5.5 and is bactericidal to other lactobacilli, sensitive to heat (30 min at 100°C), and encoded by chromosomal determinants. Helveticin J was purified by ammonium sulfate precipitation and gel filtration and identified as a 37,000-Da protein (44). The genes for helveticin J (*hlv*) have been cloned by Joerger and Klaenhammer (45), who reported the identification and expression of both the structural and immunity genes in transformants. Their work (45) represents the first cloning and expression of bacteriocin genes from lactobacilli.

Two bacteriocins produced by strains of *Lb. acidophilus* have been studied extensively. The heat-stable bacteriocin LaF is produced by *Lb. acidophilus* 11088 (NCK 88) and is bactericidal to *Lb. delbrueckii* ssp. *bulgaricus* 1489, *Lb. delbrueckii* ssp. *lactis* (formerly *Lactobacillus leichmannii*) ATCC 4797, *Lb. helveticus* 87, *Lb. acidophilus* 6032, *Lb. fermentum* 1750, and *Ent. faecalis* 19433 (57, 58). Muriana and Klaenhammer (58) purified LaF from MRS broth cultures of *Lb. acidophilus* 11088 by ammonium sulfate precipitation, gel filtration, and HPLC. Analysis of LaF by SDS-PAGE indicated that activity was associated with a 2500-Da band (50). Amino acid sequence analysis of the purified protein identified 25 N-terminal residues; composition analysis suggested that the protein contained 56 amino acids with a molecular size of approximately 6300 Da (58). The discrepancy in estimated size was resolved by cloning and characterization of the LaF gene (*laf*) (59). The *laf* determinant was detected with a 63-base oligonucleotide probe that is homologous to the N-terminal *laf* amino acid sequence (59). The probe hybridized to a 2.2-kb fragment of a 110-kb LaF plasmid in the LaF-producing culture, *Lb. acidophilus* T143 (57). Muriana and Klaenhammer (59) constructed the plasmid vector pTRK159 that is capable of replicating in both *E. coli* and lactobacilli. The 2.2-kb fragment from the LaF plasmid was subcloned into the vector and transformed by electroporation into strains of *Lb. acidophilus* that did not produce LaF. Two transformants harbored the 2.2-kb fragment, produced LaF, and were immune to it. Analysis of the DNA sequence identified at least three ORF. One ORF corresponded to a 75-amino acid protein with an N-terminal extension that is removed during processing. The 57-amino acid sequence deduced from the remainder of the *laf* gene correlated with the size that was predicted by composition analysis and confirmed a size of 6300 Da for LaF (59). A subsequent report (3) indicates that the LaF structural gene is one of four adjacent ORF with a common promoter and transcription terminator that appear to be within a single operon.

LACTACIN B PRODUCED BY *LB. ACIDOPHILUS* N2

Lactacin B is a peptide bacteriocin produced by *Lb. acidophilus* N2 (7, 8). Lactacin B is sensitive to pronase and to proteinase K and is stable to chaotropic agents (urea and SDS) and heat (100°C for 3 or 30 min at pH 5). Lactacin B possesses a more limited spectrum than Laf and inhibits only *Lb. helveticus*, *Lb. delbrueckii* ssp. *lactis*, and *Lb. delbrueckii* ssp. *bulgaricus* (7, 8). Lactacin B was purified by ion-exchange chromatography, ultrafiltration in urea, and sequential gel filtrations in urea and SDS (8). Activity, but no corresponding protein band, was detected after SDS-PAGE, suggesting that lactacin B was either present in undetectable amounts or could not be silver stained. The extreme activity losses (typically 99.6%) encountered during purification (8) precluded further biochemical and immunological studies.

Efforts to characterize lactacin B have continued. In Gram-negative bacteriocinogenic species, synthesis of inducible bacteriocins occurs after activation of the SOS repair system in response to DNA-damaging agents (69). In our laboratory, Hughes (39) hypothesized that

bacteriocin production might be similarly stimulated by some environmental factor, possibly a signal from sensitive cells. To examine effects on lactacin B production, Hughes and Barefoot (40) associatively cultured the producer *Lb. acidophilus* N2 in a bench top fermentation system with the sensitive indicator *Lb. delbrueckii* ssp. *lactis* ATCC 4797 and monitored bacteriocin activity and pH. Activity (Figure 1) typically was detected in associative cultures during early logarithmic growth (within 5 h) and increased to a maximum of 6400 AU (activity units)/ml within an additional 6 h. No activity was detected in pure cultures of *Lb. acidophilus* N2 when pH was not controlled. When the pH of both cultures was compared (Figure 1), the initial pH of associative cultures was .2 units higher than for producer cultures alone. The pH in both cultures dropped at an equivalent rate for about 6 h; the pH of the pure culture decreased more rapidly thereafter. These data did not eliminate

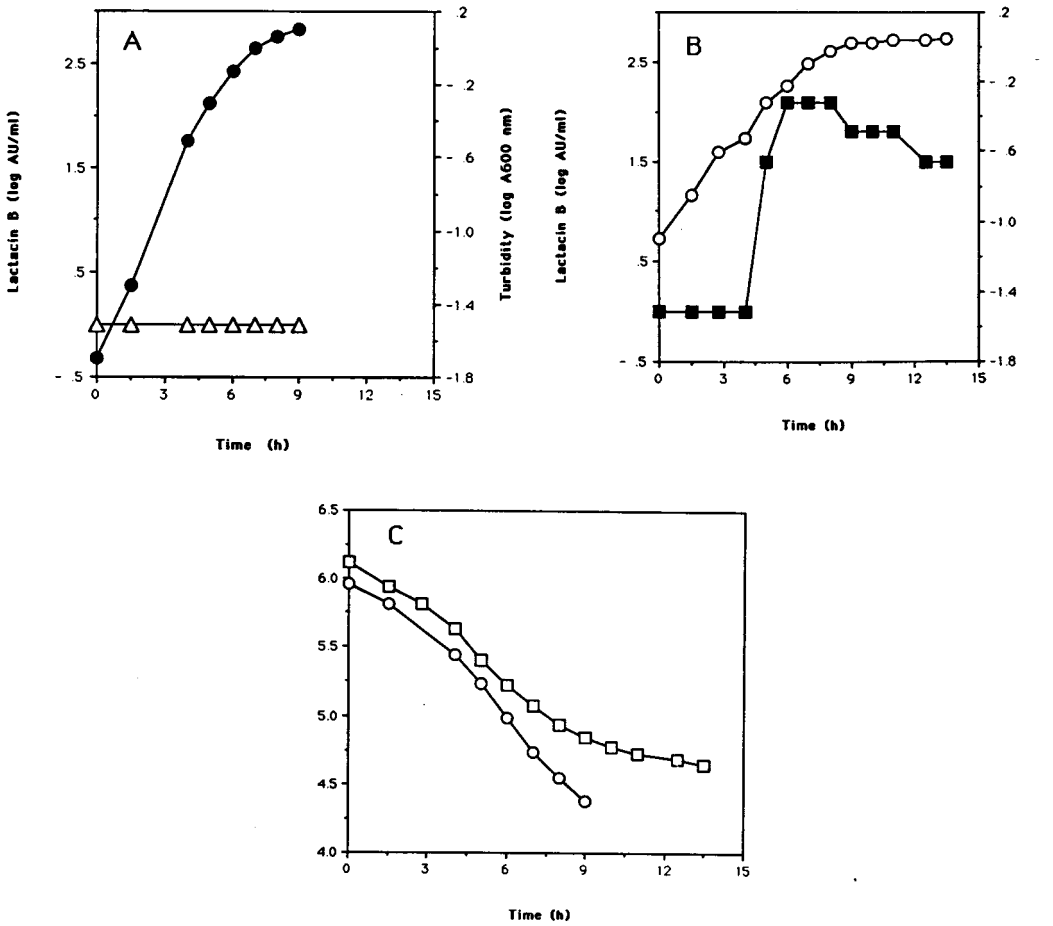

Figure 1. Lactacin B production in producer and associative cultures without pH control. *Lactobacillus acidophilus* N2 was propagated (inoculum, 1% of a 20-h MRS broth culture) alone and in association with *Lactobacillus delbrueckii* ssp. *lactis* ATCC 4797 (1% inoculum, 25-h MRS broth culture) in sterile MRS broth (500 to 600 ml; BBL Microbiology Systems, Cockeysville, MD), maintained at 37°C and continuously agitated (50 to 100 rpm) in a 1-L fermentation system. Cell turbidity was determined spectrophotometrically as absorbance at 600 nm ($A_{600\ nm}$) lactacin B activity was assayed and converted to $\log_{10}$ activity units per milliliter, and pH was monitored. A) N2 alone: culture turbidity as $A_{600\ nm}$ (●); lactacin B activity as $\log_{10}$ activity units per milliliter (△). B) N2 and 4797 in associative culture: culture turbidity in $\log_{10\ nm}$ (○); lactacin B activity in $\log_{10}$ activity units per milliliter (■). C) Culture pH of N2 alone (○) and of N2 and 4797 grown in association (□).

the possibility that increased production of lactacin B in associative cultures was partially related to pH.

To eliminate pH effects, cultures of the lactacin B producer N2 (Figure 2A) and N2 in association with 4797 (Figure 2B) were maintained at pH 6.0 and examined for inhibitory activity. Equivalent maximum activity (25,600 AU/ml) was detected in both associative and pure producer cultures. Associative growth of N2 and 4797 resulted in early appearance of activity. Activity in associative cultures was detected within 5 h; in pure cultures of the producer N2, activity typically was detected after 9 h. These results suggest that early production of lactacin B in associative culture was not due to pH effects (39, 40).

To permit differential enumeration of producer and indicator populations during associative culture, spontaneous streptomycin-

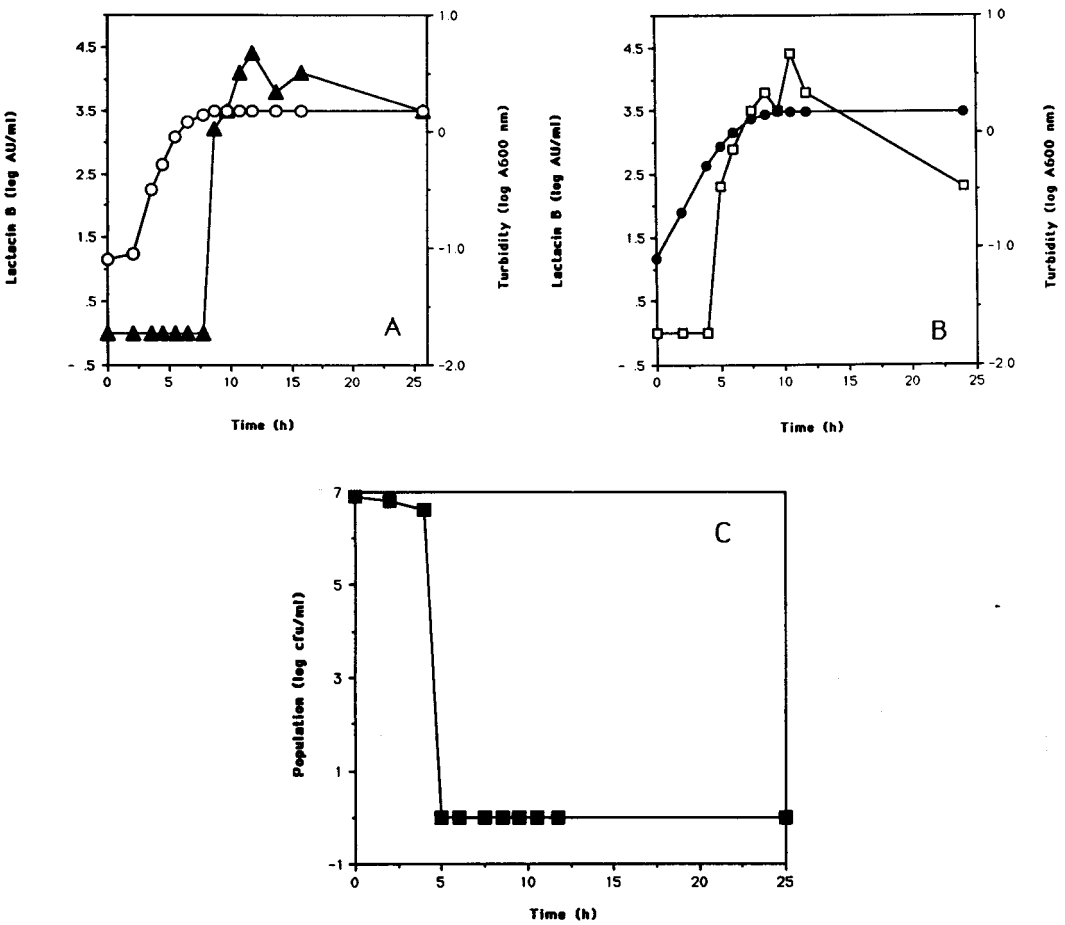

Figure 2. Lactacin B production in producer and associative cultures maintained at pH 6. *Lactobacillus acidophilus* N2 was propagated alone (1% inoculum, 20-h MRS broth culture) and in association with *Lactobacillus delbrueckii* ssp. *lactis* ATCC 4797 (1% inoculum, 25-h MRS broth culture) in a 1-L fermentation system containing sterile MRS broth (BBL Microbiology Systems, Cockeysville, MD) maintained at 37°C, pH 6.0, and continuously agitated (50 to 100 rpm). Culture turbidity was monitored spectrophotometrically at 600 nm ($A_{600\ nm}$), and lactacin B activity was assayed and converted to $\log_{10}$ activity units (AU) per milliliter. A) N2 alone: cell turbidity as $A_{600\ nm}$ (○); lactacin B activity in $\log_{10}$ AU per milliliter (▲). B) Associative culture: turbidity as $A_{600\ nm}$ (●); lactacin B activity in $\log_{10}$ AU per milliliter (□). C) Viability in $\log_{10}$ colony-forming units per milliter (■) of *Lb. delbrueckii* ssp. *lactis* ATCC 4797 in associative cultures was determined by plating on MRSS agar (MRS broth containing 1000 μg/ml of streptomycin sulfate and 1.5% granulated agar).

resistant (Str^r) mutants (1000 μg/ml) of the sensitive indicator *Lb. delbrueckii* ssp. *lactis* ATCC 4797 were selected. Mutants retained Str^r throughout growth. The viability of Str^r indicator cells in both associative cultures without pH control and those maintained at pH 6.0 was monitored. Cells of 4797 grown in association with the producer N2 typically died within 5 h (Figure 2C). Inhibitory activity in associative cultures increased shortly after the death of 4797. Maximum activity always appeared well after the death of the indicator population. No activity was ever detected in pure cultures of *Lb. delbrueckii* ssp. *lactis* ATCC 4797.

Culturing the lactacin B producer *Lb. acidophilus* N2 associatively, with the sensitive indicator *Lb. delbrueckii* ssp. *lactis* 4797, either at pH 6.0 or in cultures without pH control, resulted in early, increased production of an inhibitor with characteristics identical to those of lactacin B. Like lactacin B (7, 8), the associatively produced inhibitor was stable to heat and chaotropic agents. In addition, the inhibitor was active against *Lb. delbrueckii* ssp. *bulgaricus* 1489, *Lb. helveticus* 87, and *Lb. delbrueckii* ssp. *lactis* strains 970 and 4797; lactacin B is active against the same cultures (7, 8). These results agree with those of previous reports (8) that specify that lactacin B was not detected in producer cultures grown in MRS broth (pH 6.5) without pH control. However, associative producer and indicator cultures propagated without pH control contained inhibitory activity (1600 to 6400 AU/ml). Two observations provided evidence that the associatively produced inhibitor was synthesized by *Lb. acidophilus* N2. No activity was ever detected in pure cultures of the indicator *Lb. delbrueckii* ssp. *lactis* 4797, and associatively cultured indicator cells were killed before inhibitory activity was detected. These data support the conclusion that the inhibitor produced in associative cultures was lactacin B.

The initial number of indicator cells added to associative cultures affected lactacin B production (40). Highest activity was detected when initial producer numbers (8.1×10^6 cfu/ml) approximated indicator numbers (4×10^6 to 1×10^7 cfu/ml). Decreasing or increasing numbers of indicator cells diminished the effect on lactacin B production. These results suggest that some minimum concentration of indicator cells affected production of lactacin B. The failure of higher cell concentrations to enhance production of lactacin B may be because *Lb. delbrueckii* ssp. *lactis* 4797 grew faster than *Lb. acidophilus* N2 and reached higher populations than the producer culture when higher number of cells were added.

TABLE 4. Effect of treatments of cells of *Lactobacillus delbrueckii* ssp. *lactis* ATCC 4797 on production of lactacin B by *Lactobacillus acidophilus* N2.[1]

Treatment	(log_{10} AU/ml)[3]
Broth culture	4.1
Washed cells	3.8
Cell-free supernate	<0
MRS Broth	<0
Lipase,[2] 1 mg/ml; 37°C, 1 h	4.1
Lysozyme, 4 mg/ml; 37°C, 1 h	4.4
Protease, 1 mg/ml, 37°C, 1 h	<0

[1]Source: (39).

[2]Approximately 7.9×10^8 cells of *Lb. delbrueckii* ssp. *lactis* 4797 were pelleted by centrifugation, resuspended in .3 m*M* phosphate buffer, pH 7.2, containing the indicated concentration of enzyme, and held as indicated. Cells were pelleted by centrifugation and resuspended in the buffer (1 ml); 50 μl of each cell suspension were added to separate 10-ml tubes of MRS broth containing *Lb. acidophilus* N2 (8.4×10^6 cfu/ml).

[3]Activity units of lactacin B.

Attempts were made to locate the moiety affecting production of lactacin B (40). Cells of the indicator culture *Lb. delbrueckii* ssp. *lactis* ATCC 4797, but not its cell-free culture supernates, stimulated production of lactacin B (Table 4), suggesting that the responsible agent was cell-associated. Addition of 4797 cells killed by a mild heat treatment (55°C, 1 d) to the producer culture enhanced lactacin B production; addition of cells killed by autoclaving had no effect (Chen and Barefoot, 1991, unpublished data). Indicator cells were treated separately with protease, lipase, or lysozyme (at 500 μg of enzyme/ml, 37°C, 1 h; Sigma Chemical Co., St. Louis, MO) before being added to associative cultures. Cells treated with protease failed to enhance production, suggesting that the enhancer was proteinaceous. Cells treated with lipase or lysozyme retained their ability to enhance bacteriocin production in associative cultures at uncontrolled pH; these data suggest that the enhancer

TABLE 5. Characteristics of bacteriocins of dairy propionibacteria.

	Propionicin PLG-1	Jenseniin G
Producer	*Propionibacterium thoenii* P127 (ATCC 4874)	*Propionibacterium jensenii* P126 (ATCC 4872)
Bacteriocin source	Sodium lactate agar cultures	Sodium lactate agar and broth cultures
Enzyme sensitivity	Protease, pronase E, pepsin, trypsin, chymotrypsin	Proteinase K, pronase E, chymotrypsin, type 14 protease
Size	>150,000 and 10,000 Da	Retained in 12,000 molecular weight cutoff dialysis tubing.
Heat stability	80°C, 30 min	100°C, 15 min
Active against	Related propionibacteria, pediococci, lactobacilli, lactococci; *Campylobacter*, *Pseudomonas*, *Vibrio* spp.; assorted fungi	Related propionibacteria, lactobacilli, lactococci
Adsorbs to	*Propionibacterium acidipropionici* P5	*Lactobacillus delbrueckii* ssp. *lactis* 4797, not *P. acidipropionici* P5
Action	Bactericidal to *P. acidipropionici* P5	Bacteristatic to *P. acidipropionici*; bactericidal to *Lb. delbrueckii* ssp. *lactis* 4797.
Source	(51)	(30)

was neither lipid nor N-acetyl muramic acid. Hughes and Barefoot (40) concluded that the factor that mediated enhanced production of lactacin B was a cell-associated proteinaceous agent. Whether the stimulatory factor behaves similarly to inducers of bacteriocins produced by Gram-negative bacteria (69, 84) has not yet been determined. Clearly, genetic effects or effects at subsequent steps in protein translation, processing, or excretion could increase production of lactacin B. Purification and further characterization of the enhancing moiety are ongoing.

The insensitivity of lactacin B to chaotropic agents (8) has been used as the basis of an alternate purification protocol (60). In brief, the protocol was as follows. Eight liters of a 9-h semidefined broth culture of *Lb. acidophilus* N2 containing lactacin B were adjusted to pH 5.0, centrifuged, and filtered to remove cells. The filtered medium was concentrated by lyophilization, resuspended in 6 *M* urea, and passed through a 50,000-molecular weight cutoff (MWCO) ultrafiltration membrane to remove large contaminating molecules. The resulting filtrate was concentrated on a 3000-MWCO ultrafiltration membrane. The concentrate was dialyzed and subjected to preparative isoelectric focusing (pH 3 to 10 gradient) with a Rotofor™ (BioRad Laboratories, Richmond, CA). Fractions eluting at or above pH 5.8 were pooled, concentrated, and analyzed by SDS-PAGE. Lactacin B activity was associated with a single protein band corresponding to a molecular size of 8100 Da (60). Sequencing was not accomplished, because of blockage of the N-terminal amino acid (60). Lactocin S, a bacteriocin produced by *Lb. sake*, also had a blocked amino terminus (56).

BACTERIOCINS PRODUCED BY PROPIONIBACTERIA

Dairy propionibacteria include *Propionibacterium freudenreichii*, *Propionibacterium jensenii*, *Propionibacterium thoenii*, and *Propionibacterium acidipropionici* (13). In addition to propionic acid, acetic acid, and diacetyl, dairy propionibacteria produce both bacteriocins and the uncharacterized broad-spectrum inhibitor, Microgard™ (Wesman Foods, Beaverton, OR). Microgard™ is the pasteurized product of the fermentation of grade A skim milk by *Propionibacterium freudenreichii* ssp. *shermanii* and is added to more than 30% of the cottage cheese in the United States (15). Inhibitory actions of Microgard™ have been attributed to diacetyl, propionic, acetic, and lactic acids and to a heat-stable, 700-Da peptide (15); however, no reports characterizing the inhibitor have been published. Like propionic acid (42), Microgard™ inhibits most Gram-negative bacteria and some fungi (4); however, the relative contributions of organic acids and protein to inhibition have not been identified.

Grinstead (29) surveyed 150 strains and identified several cultures, including *P. jen-*

senii P126 (ATCC 4827) and *P. thoenii* P127 (ATCC 4874) that inhibit related dairy propionibacteria. The bacteriocin propionicin PLG-1 produced by *P. thoenii* P127 was characterized by Lyon and Glatz [Table 5; (51)]. Propionicin PLG-1 is protease-sensitive and is adsorbed by, and bactericidal to, sensitive propionibacteria. Propionicin first was isolated from soft sodium lactate agar cultures of *P. thoenii* P127 by ammonium sulfate precipitation and gel filtration (51). A more recent report (52) indicates that propionicin is produced maximally in broth producer cultures maintained at pH 7. Propionicin has been purified to homogeneity by precipitation with ammonium sulfate, ion-exchange chromatography, and isoelectric focusing and is a 10000-Da protein with an isoelectric point of 8.8 (52). Propionicin is stable from pH 3 to 9 but is rapidly inactivated at pH ≥10 (51). Treatments at temperatures greater than 80°C destroy propionicin activity. Partially purified PLG-1 displays unusually broad activity against *P. thoenii*, *P. jensenii*, and *P. acidipropionici*, lactic acid bacteria, yeasts and molds, and Gram-negative species, including single strains of *Campylobacter jejuni*, *Vibrio parahaemolyticus*, and pseudomonads (51). Propionicin is unique in that no other bacteriocins produced by dairy cultures are active against Gram-negative species.

Grinstead and Barefoot (30, 31) characterized the bacteriocin produced by *P. jensenii* P126 (Table 5). Jenseniin G is produced in both sodium lactate agar and broth cultures, is sensitive to proteases, and is retained by dialysis tubing with nominal exclusion limits of 12,000 Da. Unlike propionicin PLG-1 (51), jenseniin G is unaffected by treatment at 100°C for 20 min (30). Jenseniin is bacteriostatic to *P. acidipropionici* P5 and bactericidal to *Lb. delbrueckii* ssp. *lactis* ATCC 4797 (30). In contrast to the broad spectrum identified for propionicin PLG-1 (51), activity of jenseniin was confined to dairy propionibacteria, lactococci, and lactobacilli that are typically present in Swiss cheese (30). Additional studies (31) indicated that jenseniin G was stable from pH 3 to 11 and insensitive to treatment with .5% SDS or 4 *M* urea. Further purification and characterization of jenseniin G is in progress.

FUTURE OF BACTERIOCINS

Numerous bacteriocins have been identified in dairy cultures. These bacteriocins can be divided into three categories, including the large, heat-labile proteins, such as helveticin J (44, 45) and caseicin 80 (70, 71); small, usually heat-stable, nonlanthionine-containing proteins or peptides, such as LaF (57, 58, 59), lactacin B (8, 9), and lactococcin A (37, 83, 90, 91); and the lanthionine-containing peptides, nisin (41) and lacticin 481 (64, 67). Broadly inhibitory proteins–the lantibiotic nisin (41) and the heat-labile peptide propionicin PLG-1 (51, 52) of as yet unknown composition–may be found in several categories. Additional molecular information about bacteriocins in all categories will be necessary to permit construction of bacteriocins with optimal heat stability and inhibitory properties. In addition, only some bacteriocins have been assessed for inhibition of spoilage organisms or pathogens in food systems (10, 21, 34, 76, 79), and much additional applications information is needed. As indicated by Daeschel (16), bacteriocins used as food preservative must be nontoxic, stable, and highly active; have broad activity; have no adverse effects on sensory characteristics of the food; and be inexpensive and simple to use. In addition, to facilitate approval by the Food and Drug Administration and other regulatory agencies, bacteriocins for use in food systems must be well characterized biochemically and genetically. Clearly, much work on bacteriocins produced by dairy cultures remains.

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